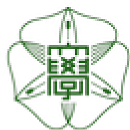
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1 Original Article

2

3 Title: Simultaneous evaluation of plasma membrane integrity, acrosomal integrity, and

4 mitochondrial membrane potential in bovine spermatozoa by flowcytometry

5

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14 Running head: Bull sperm evaluation by flowcytometry

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20

21

Summary

The present study aimed to develop an objective evaluation procedure to estimate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bull spermatozoa simultaneously by flowcytometry. Firstly, we used frozen-thawed semen mixed with 0%, 25%, 50%, 75% and 100% dead spermatozoa. Semen was stained using three staining solutions: SYBR-14, propidium iodide (PI), and phycoerythrin-conjugated peanut agglutinin (PE-PNA), for the evaluation of plasma membrane integrity and acrosomal integrity. Then, the characteristics evaluated by flowcytometry and by fluorescent microscopy were compared. In terms of the results, the characteristics of spermatozoa (viability and acrosomal integrity) evaluated by flowcytometry and by fluorescent microscopy were similar. Secondly, we attempted to evaluate plasma membrane integrity, acrosomal integrity, and also mitochondrial membrane potential of spermatozoa by flowcytometry using conventional staining with three dyes (SYBR-14, PI, and PE-PNA) combined with MitoTracker Deep Red (MTDR) staining (quadruple staining). Then, the spermatozoon characteristics evaluated by flowcytometry using quadruple staining were compared with those of staining using SYBR-14, PI, and PE-PNA and staining using SYBR-14 and MTDR. From the obtained results, there were no significant differences in all characteristics (viability, acrosomal integrity, and mitochondrial membrane potential) evaluated by quadruple staining and the other procedures. In conclusion, quadruple staining using SYBR-14, PI, PE-PNA, and MTDR for flowcytometry can evaluate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bovine spermatozoa simultaneously.

Keywords: Acrosomal integrity, Bovine spermatozoa, Flowcytometry, Mitochondrial membrane potential, Spermatozoon viability

49 **Introduction**

50 Artificial insemination (AI) using frozen-thawed bull semen is a generally used
51 technique for the reproduction of dairy and beef cattle. It was reported that the
52 improvement of frozen-thawed semen quality, such as motility, malformation, and
53 concentration of spermatozoa in semen, was positively correlated with the pregnancy
54 rate (Brito *et al.*, 2002). Semen collected from bulls is diluted, cooled, and frozen for
55 long-term storage until insemination into the female genital tract. All processing steps
56 of semen cryopreservation may induce damage to the plasma membrane and cellular
57 structure of spermatozoa (Hammerstedt *et al.*, 1990; Silva & Gadella, 2006; Watson,
58 2000). Therefore, the evaluation of spermatozoon characteristics by laboratory assays
59 is very important to achieve a high pregnancy rate by AI using frozen-thawed semen.
60 There are several reports dealing with criteria for the evaluation of various
61 spermatozoon characteristics: motility, viability, morphological abnormality, and
62 organelle functions (Den Daas *et al.*, 1998; Linford *et al.*, 1976; Söderquist *et al.*, 1991;
63 Thomas *et al.*, 1998). However, most of the evaluation methods in these reports are
64 subjective because they are achieved by microscopic observation and the obtained
65 results may fluctuate depending on the practitioner. Therefore, objective and
66 quantitative methods should be chosen for the evaluation of spermatozoon
67 characteristics. Moreover, it is thought that the results from any single laboratory
68 assay will not effectively estimate the fertilizing potential of a semen sample (Graham
69 & Mocé, 2005); therefore, combined multiple assays are necessary to estimate the
70 characteristics of spermatozoa more accurately.

71 Recently, flowcytometry has been used as an objective tool for evaluating multiple
72 characteristics of a large number of spermatozoa (Vincent *et al.*, 2012). Nagy *et al.*
73 (2003) demonstrated that triple staining by SYBR-14, propidium iodide (PI), and
74 phycoerythrin-conjugated peanut agglutinin (PE-PNA) was effective for evaluation of

75 the viability and acrosomal integrity of bovine spermatozoa simultaneously. In
76 addition, Thomas *et al*. (1998) proved that mitochondrial membrane potential of
77 spermatozoa could be assessed by flowcytometry using JC-1 as a probe. If these two
78 methods can be combined, we can evaluate 3 items, viability, acrosomal integrity and
79 mitochondrial membrane potential, of spermatozoa simultaneously and it is possible to
80 obtain more detailed information about each spermatozoon. However, the
81 combination of these reagents for flowcytometry cannot be achieved because such
82 staining uses the same excitation (488 nm) and the broad-emission spectral properties of
83 JC-1 (green, 510-520 nm, and red-orange, 590 nm) overlap with SYBR-14 (517 nm).
84 It is also difficult to distinguish JC-1 from PI (617 nm) and PE-PNA (580 nm) by
85 flowcytometry. Celeghini *et al*. (2007) reported simultaneous evaluation of viability,
86 acrosome integrity, and mitochondrial membrane potential using fluorescent microscopy.
87 However, their method cannot be applied to flowcytometry because they used PI and
88 JC-1. Hallap *et al*. (2005) reported that spermatozoa having high mitochondrial
89 membrane potential, which were judged by double staining with SYBR-14 and
90 MitoTracker Deep Red (MTDR), showed high motility. An excitation laser of MTDR
91 (640 nm) is different from those of SYBR-14, PI, and PE-PNA (488 nm). Moreover,
92 MTDR is known as a highly specific probe for mitochondria (Martínez-Pastor *et al.*,
93 2010). Therefore, MTDR is a candidate for evaluating mitochondrial membrane
94 potential simultaneously with triple staining mentioned above.

95 In the present study, we aimed to develop an objective evaluation procedure for
96 plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential
97 of spermatozoa simultaneously using flowcytometry after staining with SYBR-14, PI,
98 PE-PNA, and MTDR.

99

100 Materials and Methods

101 **Semen**

102 Frozen semen, which was diluted with egg yolk-Tris-glycerol (6%) extender and packed
103 in 0.5-ml straw, derived from the same ejaculates of 5 Holstein bulls donated from
104 Genetics Hokkaido Association (Sapporo, Japan), were used for this study. The semen
105 was thawed at 37˚C for 45 sec in water and expelled into a 1.5-ml tube. The thawed
106 semen was used for different staining, as follows. Dead spermatozoa used in
107 experiment 1 were prepared by thawing at 37˚C in water and refreezing in liquid
108 nitrogen twice.

109

110 **Double staining for evaluation of mitochondrial membrane potential**

111 Staining solution was prepared as described in a previous study (Hallap *et al.*, 2005).
112 In brief, 100 µl of MTDR (final concentration 10 nM; M22426, Life Technologies,
113 Carlsbad, CA, USA), 1 µl of SYBR-14 (final concentration 100 µM; L-7011
114 LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR, USA) and 800 µl of
115 Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS) were
116 mixed (staining solution). Then, 100 µl of semen was mixed with staining solution
117 and warmed at 37˚C for 10 min in the dark.

118

119 **Triple staining for evaluation of viability and acrosomal integrity of spermatozoa**

120 Staining solution was prepared as described in a previous study (Nagy *et al.*, 2003).
121 Briefly, 1 µl of SYBR-14, 2.5 µl of PE-PNA (final concentration 2.5 µg/ml; GTX01509,
122 GeneTex, Irvine, CA, USA), 5 µl of PI (final concentration 12 µM; L-7011,
123 LIVE/DEAD Sperm Viability Kit, Molecular Probes), and 900 µl of DPBS were mixed
124 (staining solution). Then, 100 µl of semen was mixed with staining solution and
125 warmed at 37˚C for 10 min in the dark.

126

127 **Quadruple staining for simultaneous evaluation of viability, acrosomal integrity,**
128 **and mitochondrial membrane potential of spermatozoa**

129 The same volume and types of fluorescent dye as in the triple staining along with 100 µl
130 of MTDR solution were added to 800 µl of DPBS (staining solution). Then, 100 µl of
131 semen was mixed with the staining solution and warmed at 37˚C for 10 min in the dark.
132

133 **Analysis by flowcytometry**

134 After staining, 10 µl of 10% (v/v) formaldehyde (final concentration 0.1%) was added
135 to all samples (1,000 µl) to immobilize the living spermatozoa in the staining solution,
136 as described in a previous study (Harrison and Vickers, 1990). Subsequently, 100 µl of
137 stained sample was mixed with 400 µl of DPBS and subjected to flowcytometry.
138 Sperm suspensions were run through a flowcytometer (FACS VerseTM, BD Biosciences,
139 San Jose, CA, USA). SYBR-14, PI, and PE-PNA were excited using a 488-nm
140 excitation laser and detected in an FITC filter (527/32 nm), PE-filter (586/42 nm), and
141 Per-CP-Cy5.5 filter (700/54 nm), respectively. MTDR was excited at 640 nm and
142 detected in an APC filter (660/10 nm). Flowcytometric gating of spermatozoa was
143 performed as reported by Hallap *et al.* (2005) and Nagy *et al.* (2003). The gating of
144 quadruple staining was performed as described in Fig. 1. Briefly, particles stained with
145 SYBR-14 or PI were judged as spermatozoa (Fig. 1 A). Spermatozoa were divided
146 into 2 groups (live and dead) by PI emission (Fig. 1 B) and then each group was gated
147 by PE-PNA and MTDR (Figs. 1 C and D). Fluorescent data of all events were
148 collected until 10,000 gated events were counted. Triplicate measurements per sample
149 were conducted and the average was used as a value of the sample.
150

151 **Analysis by fluorescent microscopy**

152 After staining and immobilization, an 8-µl sample was loaded on a slide, coverslipped,

and evaluated immediately under a fluorescent microscope (ECLIPSE Ci, Nikon, Tokyo, Japan) equipped with a B-2A filter (excitation 450-490 nm and emission >520 nm) and a G2-A filter (excitation 510-560 nm and emission >590 nm) at ×400 magnification. Microscopic examination was mainly conducted by using a B-2A filter. A G2-A filter was used for the evaluation of plasma membrane integrity when PI emission was not clear. Two hundred spermatozoa per slide were examined and classified based on the fluorescence emitted from each probe (Table 1). Three slides per sample were examined and the average was used as the value of the sample.

Evaluation of spermatozoon characteristics

Spermatozoon characteristics estimated by flowcytometry are described in Table 1. Briefly, when spermatozoa were stained with PI, they were evaluated as dead because the damage to the plasma membrane allowed the PI to penetrate inside them. When acrosome was stained with PE-PNA, it was evaluated as damaged. When the midpiece of spermatozoon was stained with MTDR, it was evaluated that the spermatozoa had high mitochondrial membrane potential.

Spermatozoon characteristics evaluated by fluorescent microscopy are expressed in Fig. 2. Spermatozoa stained with PI were evaluated as dead in the same way as by flowcytometry. When acrosomal region of spermatozoon was stained with PE-PNA, it was evaluated as damaged acrosome. Some spermatozoa were stained with PE-PNA intermediately (Fig. 2C), they were also judged as spermatozoon with a damaged acrosome.

Experimental design

In experiment 1, frozen-thawed semen was mixed with 0%, 25%, 50%, 75%, and 100%

dead spermatozoa. These samples were subjected to triple staining. After staining, half of the sample was evaluated by flowcytometry and the other half by fluorescent microscopy; the obtained results were then compared. Semen derived from 5 bulls was used for this experiment.

In experiment 2, frozen-thawed semen derived from a bull was subjected to double, triple, and quadruple staining. Then, the results of the spermatozoon characteristics estimated by quadruple staining were compared with those of double or triple staining samples. The experiment was repeated 4 times on independent samples.

Statistical analysis

Statistical analysis was performed using JMP 9.0.2 (SAS, NC, USA). The correlation between each characteristic of spermatozoa estimated by flowcytometry and by fluorescent microscopy was analyzed by linear regression analysis. The percentages of spermatozoon characteristics examined using different equipment and different staining procedures were compared by Student's *t*-test. Differences with $P < 0.05$ were recognized as significant.

Results

Experiment 1: The viability and acrosomal integrity of spermatozoa evaluated by flowcytometry and fluorescent microscopy were significantly correlated ($r > 0.9$, $P < 0.01$), except for the live spermatozoa with a damaged acrosome ($P = 0.866$), as shown in Fig. 3. The percentages of each characteristic evaluated by flowcytometry and fluorescent microscopy are shown in Table 2. There were no significant differences in all characteristics (live spermatozoa with an intact acrosome, live spermatozoa with a damaged acrosome, dead spermatozoa with an intact acrosome, and dead spermatozoa

204 with a damaged acrosome) evaluated by the two types of equipment ($P > 0.05$). The
205 percentages of live spermatozoa with a damaged acrosome were low among the samples
206 with different mixed ratios of dead spermatozoa.
207 Experiment 2: The viability, acrosomal status, and mitochondrial membrane potential of
208 spermatozoa evaluated by flowcytometry after quadruple staining and by the other
209 staining procedures are shown in Table 3. There were no significant differences in all
210 characteristics evaluated by quadruple staining and the other procedures ($P > 0.05$).
211 By quadruple staining, more than 95% of the live spermatozoa having an intact
212 acrosome showed high mitochondrial membrane potential. In addition, more than
213 95% of dead spermatozoa having an intact acrosome showed low mitochondrial
214 membrane potential.
215
216 **Discussion**
217 In the present study, the results of spermatozoon characteristics evaluated by
218 flowcytometry and fluorescent microscopy were similar, except for live spermatozoa
219 with a damaged acrosome. High correlation may be due to the criteria of spermatozoa
220 evaluation. The count of fluorescent intensity obtained by flowcytometry indicated
221 two obvious peaks, those evaluated as negative and positive. However, PE-PNA
222 positive peak had a broad base toward the low intensity (10^3-10^4) as shown in Fig. 1E,
223 and this small peak might be a subpopulation of spermatozoa those observed as
224 intermediately stained and judged as positive under fluorescent microscopy. Therefore,
225 evaluation of spermatozoa using two equipment could evaluate spermatozoa
226 characteristics by same criteria and provide us similar results. This exception may
227 have been caused by quite a small population (< 1%) of live spermatozoa with a
228 damaged acrosome in semen. The number of spermatozoa evaluated by
229 flowcytometry was about 50 times greater than by fluorescent microscopy, which

230 examined about 200 spermatozoa (Celeghini *et al.*, 2007; Somfai *et al.*, 2002). In spite
231 of evaluating a large number of spermatozoa, quite a small population of this type of
232 spermatozoa may indicate that spermatozoa die immediately after damage to the
233 acrosome.

234 In the present study, the viability, acrosomal integrity, and mitochondrial membrane
235 potential of spermatozoa could be evaluated accurately by quadruple staining without
236 interference of fluorescent dye. The present results mean that most of the spermatozoa
237 with damage to the plasma membrane had impaired mitochondrial membrane potential,
238 even though about two-third of them had the intact acrosome. Mitochondria produce
239 ATP, which is required for housekeeping of the plasma membrane of spermatozoa (Silva
240 & Gadella, 2006). In the present study, most of the live spermatozoa had high
241 mitochondrial membrane potential, while the dead spermatozoa showed a low one.
242 Low mitochondrial membrane potential indicates a decrease or lack of ATP production.
243 A decrease of ATP production may become a cause of spermatozoon death without
244 acrosomal damage. Mitochondrial activity is crucial and correlates with the
245 fertilization ability of spermatozoa (Amaral *et al.*, 2013). In further study, the
246 relationship between fertility and spermatozoon characteristics as evaluated by
247 flowcytometry using quadruple staining should be carried out.

248 A staining method to estimate the viability of spermatozoa, acrosomal integrity, and
249 mitochondrial functions simultaneously by using four fluorescent dyes (Hoechst 33342,
250 PI, FITC-PSA, and JC-1) under a fluorescent microscope has also been reported
251 (Celeghini *et al.*, 2007). However, in this previous study (Celeghini *et al.*, 2007), only
252 hundreds of spermatozoa could be evaluated subjectively. On the other hand, the
253 method developed in the present study enables the objective estimation of more than
254 10,000 sperm by flowcytometry in a short time. This means that the characteristics of
255 spermatozoa can be evaluated more accurately and quickly than ever by our procedure.

256 In conclusion, the quadruple staining using SYBR-14, PI, PE-PNA, and MTDR for
257 flowcytometry can evaluate the plasma membrane integrity, acrosomal integrity, and
258 mitochondrial membrane potential of bovine spermatozoa simultaneously. The
259 procedure can be applied to the quality control of bovine frozen-thawed semen.

260

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264

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329
330

Figure legends

Figure 1 Gating procedure and judgement for quadruple staining analysis by flowcytometry
Items stained with SYBR-14 and propidium iodide (PI) were distinguished as spermatozoa and gated from all events (area in red line; A). Gated spermatozoa were divided into live and dead clusters (B) followed by classification into 4 groups (Q1-4) by acrosome integrity and mitochondrial membrane potential in live (C) and dead (D) spermatozoa.
Q1: damaged acrosome with low mitochondrial membrane potential, Q2: damaged acrosome with high mitochondrial membrane potential, Q3: intact acrosome with low mitochondrial membrane potential, and Q4: intact acrosome with high mitochondrial membrane potential. PE-PNA: phycoerythrin-conjugated peanut agglutinin, MTDR: MitoTracker Deep Red.
The judgement of each spermatozoon characteristic by flowcytometry depended on fluorescent intensity. Spermatozoa with low fluorescent intensity ($<10^3$) was judged as PE-PNA negative (intact acrosome) and spermatozoa with high fluorescent intensity ($\geq 10^3$) was judged as PE-PNA positive (damaged acrosome) (E).

Figure 2 The photographs of spermatozoon triple staining taken by a florescent microscopy
The head of spermatozoon stained with SYBR-14 and acrosomal region not stained with PE-PNA (A) were judged as a live spermatozoon with an intact acrosome. The head of spermatozoon stained with PI but acrosomal region not stained with PE-PNA (B) was judged as dead spermatozoon with an intact acrosome. The heads of spermatozoon stained with PI and acrosomal region stained intermediately (C) and completely with PE-PNA (D) were both judged as a dead spermatozoon with a damaged acrosome.

Figure 3 Scatter plots and regression lines of percentages of spermatozoa evaluated by flowcytometry and fluorescent microscopy. Semen from 5 bulls was used and data from the

357 same bull are indicated by the same symbol.

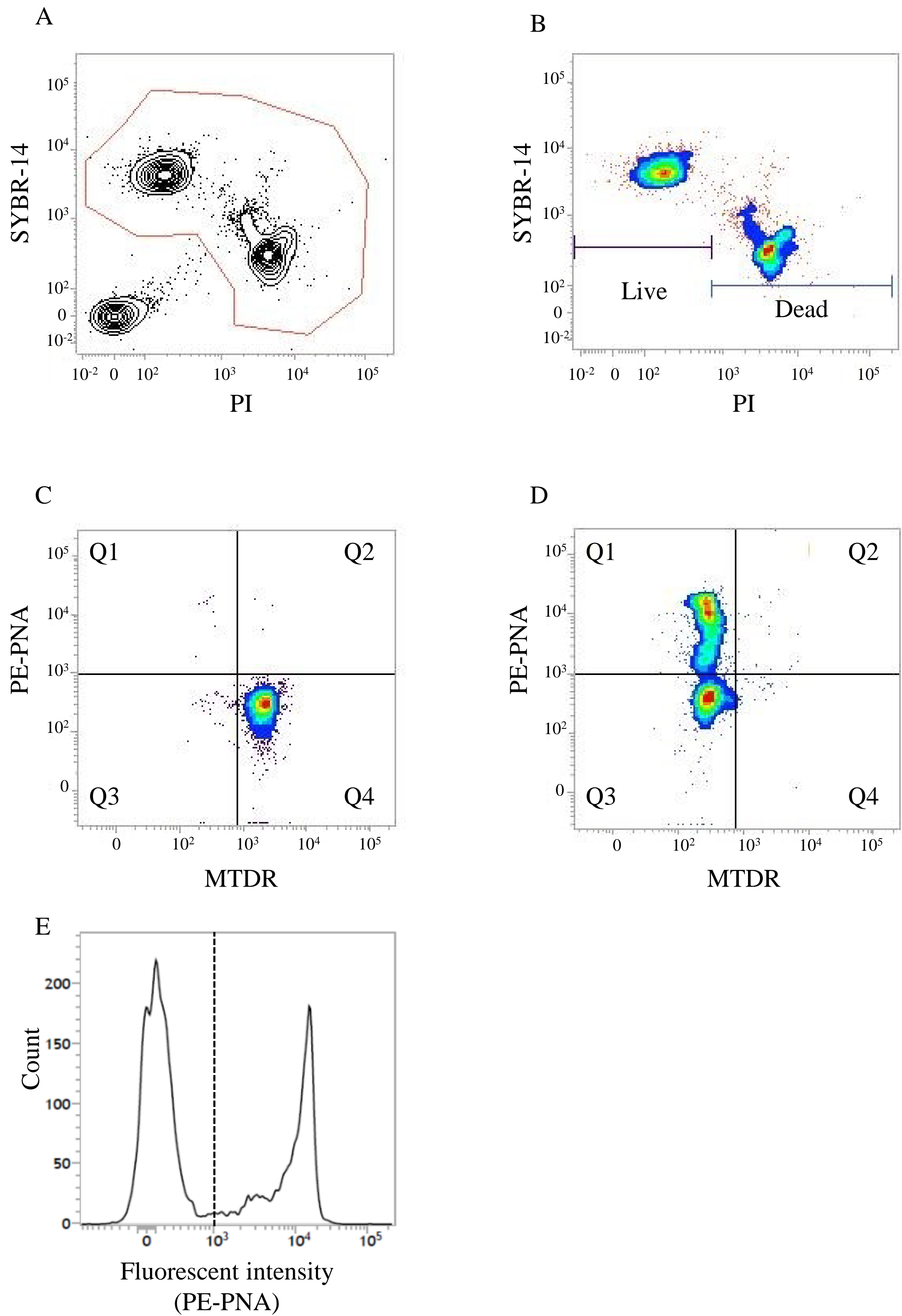

Fig. 1

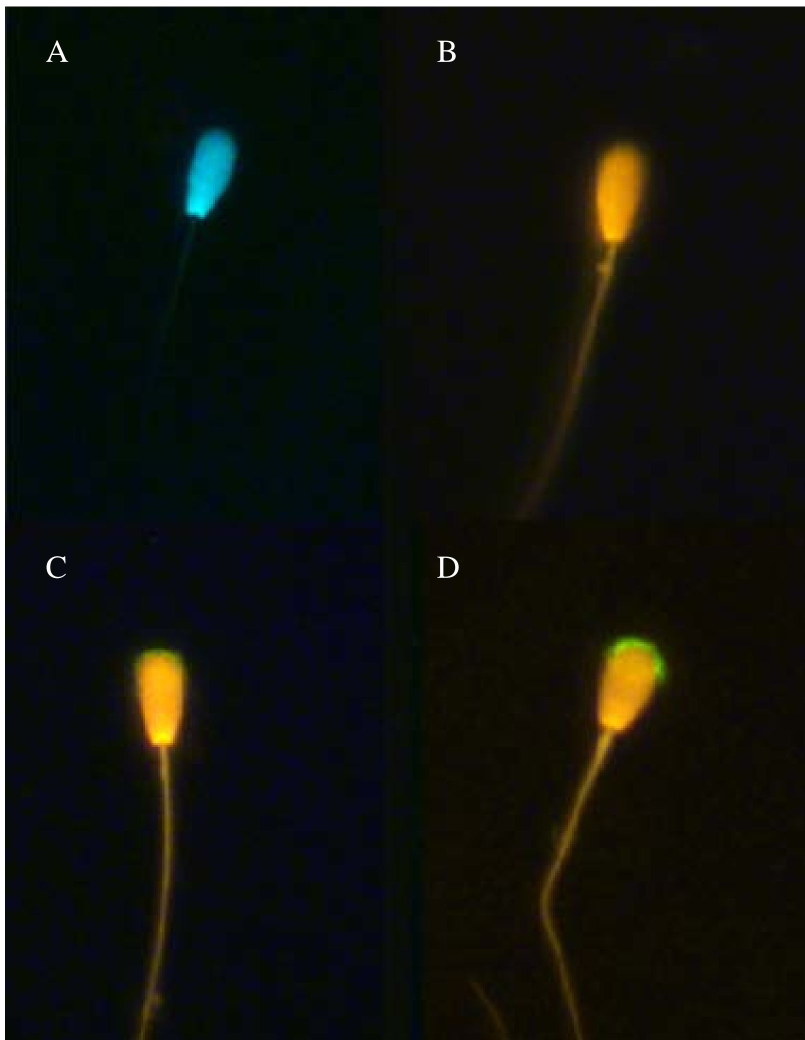

Fig. 2

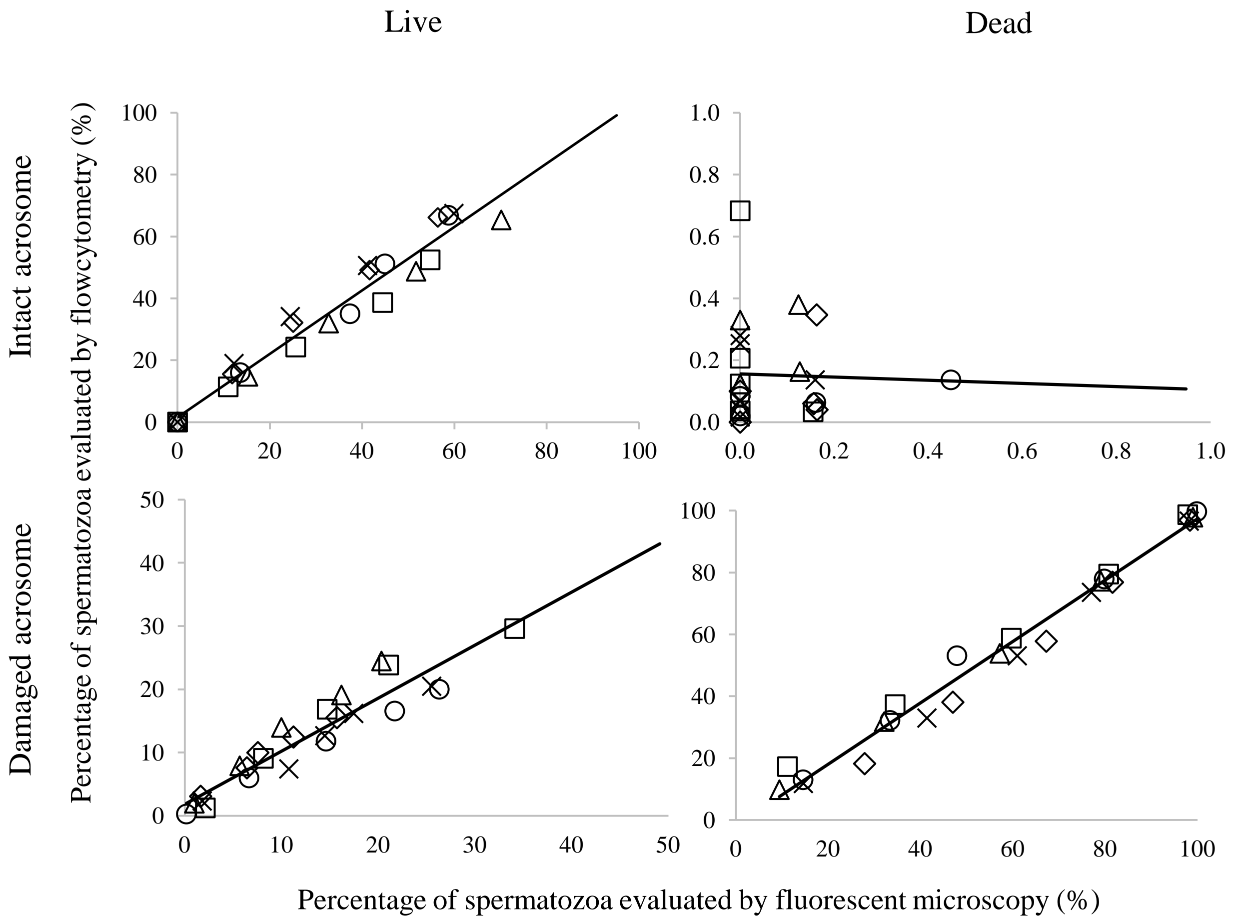

Fig. 3

Table 1 Staining patterns and evaluation of spermatozoon characteristics by each staining method

Staining procedure	Staining pattern			Spermatozoon characteristics		
	PI	PE-PNA	MitoTracker Deep Red	Viability	Acrosome	Mitochondrial membrane potential
Double	n.e.	n.e.	+	n.e.	n.e.	high
	n.e.	n.e.	-	n.e.	n.e.	low
Triple	-	-	n.e.	live	intact	n.e.
		+	n.e.		damaged	n.e.
	+	-	n.e.	dead	intact	n.e.
		+	n.e.		damaged	n.e.
Quadruple	-	-	+	live	intact	high
			-			low
		+	+		damaged	high
			-			low
	+	-	+	dead	intact	high
			-			low
		+	+		damaged	high
			-			low

+, fluorescence-positive
-, fluorescence-negative
n.e., not evaluated

7 **Table 2** Characteristics of bovine spermatozoa, the mixture of thawed semen and dead spermatozoa, evaluated by

8 flowcytometry and fluorescent microscopy after triple staining

Equipment	Spermatozoon characteristics		% of spermatozoa classified for each characteristic				
	Viability	Acrosome	1:0*	3:1*	1:1*	1:3*	0:1*
Flow cytometry	live	intact	63.7±5.6	47.7±4.6	31.5±3.8	15.3±2.4	0.0±0.0
		damaged	0.3±0.2	0.2±0.1	0.2±0.1	0.0±0.0	0.0±0.0
	dead	intact	22.0±4.7	17.6±3.8	12.9±2.0	7.6±1.0	1.8±1.0
		damaged	14.0±3.2	34.5±2.7	55.3±2.5	77.0±1.9	98.0±1.1
Fluorescent microscopy	live	intact	60.0±5.4	44.8±3.8	29.0±5.1	10.4±5.4	0.0±0.0
		damaged	0.1±0.2	0.0±0.1	0.1±0.1	0.0±0.1	0.0±0.0
	dead	intact	24.4±6.2	17.5±3.8	12.3±2.9	7.5±1.8	1.3±0.7
		damaged	15.5±6.5	35.7±7.8	58.6±6.3	79.6±1.6	98.7±0.7

9 Values are mean ± standard deviation (5 bulls/group).

10 * Mixed ratio of frozen-thawed semen and dead spermatozoa.

11 **Table 3** Spermatozoon characteristics evaluated by flowcytometry using different staining procedures

Spermatozoon characteristics			% of spermatozoon characteristics evaluated by each staining		
Viability	Acrosome	Mitochondrial membrane potential	Quadruple	Triple	Double
Live	intact	high	64.7 ± 1.5	-	-
		low	2.0 ± 0.5	-	-
		total	66.8 ± 2.3	67.0 ± 1.4	-
	damaged	high	0.1 ± 0.1	-	-
		low	0.0 ± 0.0	-	-
		total	0.1 ± 0.1	0.1 ± 0.1	-
Dead	intact	high	0.9 ± 0.4	-	-
		low	21.2 ± 1.1	-	-
		total	22.0 ± 1.8	21.7 ± 1.6	-
	damaged	high	0.4 ± 0.1	-	-
		low	10.7 ± 1.3	-	-
			11.1 ± 1.4	11.2 ± 0.4	-
Total of high mitochondrial activity			66.1 ± 1.5	-	67.9 ± 1.5
Total of low mitochondrial activity			33.9 ± 1.5	-	32.1 ± 1.5

12 Values are mean ± standard deviation (4 replicates).

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